NUCLEIC ACID LIGANDS WHICH BIND TO HEPATOCYTE GROWTH FACTOR/SCATTER FACTOR (HGF/SF) OR ITS RECEPTOR C-MET

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a divisional of United States Patent Application Serial No. 09/364,539, filed July 29, 1999, entitled "Nucleic Acid Ligands which Bind to Hepatocyte Growth Factor Scatter Factor (HGF/SF) or its Receptor C-Met," which is a continuation-in-part of United States Patent Application Serial No. 09/502,344, filed August 27, 1998, entitled "Nucleic Acid Ligands," which is a continuation of United States Patent Application Serial No. 08/469,609, filed June, 6, 1995, entitled "Method for Detecting a Target Molecule in a Sample Using a Nucleic Acid Ligand," now U.S. Patent No. 5,843,653, which is a continuation of United States Patent Application Serial No. 07/714,131, filed June 10, 1991, entitled "Nucleic Acid Ligands," now U.S. Patent No. 5,475,096, which is a continuation-in-part of United States Patent Application Serial No. 07/536,428, filed June 11, 1990, now abandoned.

FIELD OF THE INVENTION

This invention is directed towards obtaining nucleic acid ligands of hepatocyte growth factor/scatter factor (HGF) and its receptor c-met. The method used in the invention is called SELEX, which is an acronym for Systematic Evolution of Ligands by EXponential enrichment. The invention is also directed towards therapeutic and diagnostic reagents for diseases in which elevated HGF and c-met activity are causative factors.

BACKGROUND OF THE INVENTION

Hepatocyte growth factor/scatter factor (abbreviated herein as HGF) is a potent cytokine which, through interaction with its receptor c-met, stimulates proliferation, morphogenesis, and migration of a wide variety of cell types, predominantly epithelial. HGF and c-met are involved in several cellular processes involved in tumorigenesis, notably angiogenesis and motogenesis, the latter having been implicated in the migration of cells required for metastasis (reviewed in references Jiang and Hiscox 1997, Histol Histopathol. 12:537-55; Tamagnone and Comoglio

1997, Cytokine Growth Factor Rev. 8:129-42; Jiang, Hiscox *et al.* 1999, Crit Rev Oncol Hematol. 29:209-48). Interestingly, proteases that degrade the extracellular matrix also activate HGF, which in turn up-regulates urokinase type plasminogen activator (uPA) and its receptor, resulting in an activating loop feeding the invasive and migratory processes required for metastatic cancer.

HGF and the c-met receptor are expressed at abnormally high levels in a large variety of solid tumors. In addition to numerous demonstrations *in vitro* of the effects of HGF/c-met on the behavior of tumor cell lines, the levels of HGF and/or c-met have been measured in human tumor tissues (reviewed in reference Jiang 1999, Crit Rev Oncol Hematol. 29:209-48). High levels of HGF and/or c-met have been observed in liver, breast, pancreas, lung, kidney, bladder, ovary, brain, prostate, gallbladder and myeloma tumors in addition to many others.

For several of the cancer types listed above, the prognostic value of measuring HGF/c-met levels has been evaluated and found to be potentially useful for determining the progression and severity of disease. The correlative data are strongest in the case of breast cancer (Ghoussoub, Dillon *et al.* 1998, Cancer. 82:1513-20; Toi, Taniguchi *et al.* 1998, Clin Cancer Res. 4:659-64), and non-small cell lung cancer (Siegfried, Weissfeld *et al.* 1997, Cancer Res. 57:433-9; Siegfried, Weissfeld *et al.* 1998, Ann Thorac Surg. 66:1915-8).

Elevated levels of HGF and c-met have also been observed in non-oncological settings, such as hypertension (Morishita, Aoki *et al.* 1997, J Atheroscler Thromb. 4:12-9; Nakamura, Moriguchi *et al.* 1998, Biochem Biophys Res Commun. 242:238-43), arteriosclerosis (Nishimura, Ushiyama *et al.* 1997, J Hypertens. 15:1137-42; Morishita, Nakamura *et al.* 1998, J Atheroscler Thromb. 4:128-34), myocardial infarction (Sato, Yoshinouchi *et al.* 1998, J Cardiol. 32:77-82), and rheumatoid arthritis (Koch, Halloran *et al.* 1996, Arthritis Rheum. 39:1566-75), raising the possibility of additional therapeutic and diagnostic applications.

The role of HGF/c-met in metastasis has been elucidated in mice using cell lines transformed with HGF/c-met (reviewed in reference Jeffers, Rong *et al.* 1996, J Mol Med. 74:505-13). In another metastasis model, human breast carcinoma cells expressing HGF/c-met were injected in the mouse mammary fat pad, resulting in eventual lung metastases in addition to the primary tumor (Meiners, Brinkmann *et al.* 1998, Oncogene. 16:9-20). Also, transgenic

mice which overexpress HGF become tumor-laden at many loci (Takayama, LaRochelle *et al.* 1997, Proc Natl Acad Sci U S A. 94:701-6).

None of the data mentioned above provide proof of a direct causative role of HGF/c-met in human cancer, although the accumulated weight of the correlative data are convincing. However, a causal connection was established between germ-line c-met mutations, which constitutively activate its tyrosine kinase domain, and the occurrence of human papillary renal carcinoma (Schmidt, Duh *et al.* 1997, Nat Genet. 16:68-73).

Recent work on the relationship between inhibition of angiogenesis and the suppression or reversion of tumor progression shows great promise in the treatment of cancer (Boehm, Folkman *et al.* 1997, Nature. 390:404-7). In this report, it was shown that the use of multiple angiogenesis inhibitors confers superior tumor suppression/regression compared to the effect of a single inhibitor. Angiogenesis is markedly stimulated by HGF, as well as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Rosen, Lamszus *et al.* 1997, Ciba Found Symp. 212:215-26). HGF and VEGF were recently reported to have an additive or synergistic effect on mitogenesis of human umbilical vein endothelial cells (HUVECs) (Van Belle, Witzenbichler *et al.* 1998, Circulation. 97:381-90). Similar combined effects are likely to contribute to angiogenesis and metastasis.

Human HGF protein is expressed as a single peptide chain of 728 amino acids (reviewed in references Mizuno and Nakamura 1993, Exs. 65:1-29; Rubin, Bottaro *et al.* 1993, Biochim Biophys Acta. 1155:357-71; Jiang 1999, Crit Rev Oncol Hematol. 29:209-48). The amino-terminal 31 residue signal sequence of HGF is cleaved upon export, followed by proteolytic cleavage by uPA and/or other proteases. The mature protein is a heterodimer consisting of a 463 residue α-subunit and a 234 residue β-subunit, linked via a single disulfide bond. HGF is homologous to plasminogen: its α-subunit contains an N-terminal plasminogen-activator-peptide (PAP) followed by four kringle domains, and the β-subunit is a serine protease-like domain, inactive because it lacks critical catalytic amino acids. The recently solved crystal structure of an HGF fragment containing PAP and the first kringle domain indicate that this region is responsible for heparin binding and dimerization (Chirgadze, Hepple *et al.* 1999, Nat Struct Biol. 6:72-9), in addition to receptor interaction.

Human c-met protein is exported to the cell surface via a 23 amino acid signal sequence (reviewed in references Comoglio 1993, Exs. 65:131-65; Rubin 1993, Biochim Biophys Acta. 1155:357-71; Jiang 1999, Crit Rev Oncol Hematol. 29:209-48). The exported form of c-met is initially a pro-peptide which is proteolytically cleaved. The mature protein is a heterodimer consisting of an extracellular 50 kDa α-subunit bound by disulfide bonds to a 140 kDa β-subunit. In addition to its extracellular domain, the β-subunit has a presumed membrane-spanning sequence and a 435 amino acid intracellular domain containing a typical tyrosine kinase.

HGF is produced primarily by mesenchymal cells, while c-met is mainly expressed on cells of epithelial origin. HGF is very highly conserved at the amino acid level between species. This homology extends into the functional realm as observed in mitogenic stimulation of hepatocytes in culture by HGF across species, including human, rat, mouse, pig and dog. This indicates that human HGF can be used cross-specifically in a variety of assays.

Given the roles of HGF and c-met in disease, it would be desirable to have agents that bind to and inhibit the activity of these proteins. It would also be desirable to have agents that can quantitate the levels of HGF and c-met in individual in order to gather diagnostic and prognostic information.

The dogma for many years was that nucleic acids had primarily an informational role. Through a method known as Systematic Evolution of Ligands by EXponential enrichment, termed the SELEX process, it has become clear that nucleic acids have three dimensional structural diversity not unlike proteins. The SELEX process is a method for the in vitro evolution of nucleic acid molecules with highly specific binding to target molecules and is described in United States Patent Application Serial No. 07/536,428, filed June 11, 1990, entitled "Systematic Evolution of Ligands by EXponential Enrichment," now abandoned, United States Patent No. 5,475,096 entitled "Nucleic Acid Ligands", United States Patent No. 5,270,163 (see also WO 91/19813) entitled "Methods for Identifying Nucleic Acid Ligands," each of which is specifically incorporated by reference herein. Each of these applications, collectively referred to herein as the SELEX Patent Applications, describes a fundamentally novel method for making a nucleic acid ligand to any desired target molecule. The SELEX process provides a class of products which are referred to as nucleic acid ligands or aptamers,

each having a unique sequence, and which has the property of binding specifically to a desired target compound or molecule. Each SELEX-identified nucleic acid ligand is a specific ligand of a given target compound or molecule. The SELEX process is based on the unique insight that nucleic acids have sufficient capacity for forming a variety of two- and three-dimensional structures and sufficient chemical versatility available within their monomers to act as ligands (form specific binding pairs) with virtually any chemical compound, whether monomeric or polymeric. Molecules of any size or composition can serve as targets. The SELEX method applied to the application of high affinity binding involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using the same general selection scheme, to achieve virtually any desired criterion of binding affinity and selectivity. Starting from a mixture of nucleic acids, preferably comprising a segment of randomized sequence, the SELEX method includes steps of contacting the mixture with the target under conditions favorable for binding, partitioning unbound nucleic acids from those nucleic acids which have bound specifically to target molecules, dissociating the nucleic acid-target complexes, amplifying the nucleic acids dissociated from the nucleic acid-target complexes to yield a ligand-enriched mixture of nucleic acids, then reiterating the steps of binding, partitioning, dissociating and amplifying through as many cycles as desired to yield highly specific high affinity nucleic acid ligands to the target molecule.

It has been recognized by the present inventors that the SELEX method demonstrates that nucleic acids as chemical compounds can form a wide array of shapes, sizes and configurations, and are capable of a far broader repertoire of binding and other functions than those displayed by nucleic acids in biological systems.

The basic SELEX method has been modified to achieve a number of specific objectives. For example, United States Patent Application Serial No. 07/960,093, filed October 14, 1992, now abandoned, and United States Patent No. 5,707,796, both entitled "Method for Selecting Nucleic Acids on the Basis of Structure," describe the use of the SELEX process in conjunction with gel electrophoresis to select nucleic acid molecules with specific structural characteristics, such as bent DNA. United States Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands," now abandoned, United States Patent No. 5,763,177 entitled "Systematic Evolution of Ligands by Exponential Enrichment:

Photoselection of Nucleic Acid Ligands and Solution SELEX" and United States Patent Application Serial No. 09/093,293, filed June 8, 1998, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX" describe a SELEX based method for selecting nucleic acid ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. United States Patent No. 5,580,737 entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," describes a method for identifying highly specific nucleic acid ligands able to discriminate between closely related molecules, which can be non-peptidic, termed Counter-SELEX. United States Patent No. 5,567,588 entitled "Systematic Evolution of Ligands by EXponential Enrichment: Solution SELEX," describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule.

The SELEX method encompasses the identification of high-affinity nucleic acid ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved in vivo stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX process-identified nucleic acid ligands containing modified nucleotides are described in United States Patent No. 5,660,985 entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. United States Patent No. 5,580,737, supra, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH_2), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2' Modified Nucleosides by Intramolecular Nucleophilic Displacement," now abandoned, describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United States Patent No. 5,637,459 entitled "Systematic Evolution of Ligands by EXponential Enrichment: Chimeric SELEX," and United States Patent No. 5,683,867 entitled "Systematic Evolution of

Ligands by EXponential Enrichment: Blended SELEX," respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules.

The SELEX method further encompasses combining selected nucleic acid ligands with lipophilic compounds or non-immunogenic, high molecular weight compounds in a diagnostic or therapeutic complex as described in United States Patent Application Serial No. 08/434,465, filed May 4, 1995, entitled "Nucleic Acid Ligand Complexes". Each of the above described patent applications which describe modifications of the basic SELEX procedure are specifically incorporated by reference herein in their entirety.

It is an object of the present invention to obtain nucleic acid ligands to HGF and c-met using the SELEX process.

It is a further object of the invention to obtain nucleic acid ligands that act as inhibitors of HGF and c-met.

It is a further object of the invention to provide therapeutic and diagnostic agents for tumorigenic conditions in which HGF and c-met are implicated.

It is yet a further object of the invention to use nucleic acid ligands to HGF and c-met to diagnose and treat hypertension, arteriosclerosis, myocardial infarction, and rheumatoid arthritis.

It is an even further object of the invention to use nucleic acid ligands to HGF singly or in combination with other nucleic acid ligands that inhibit VEGF and/or bFGF, and/or possibly other angiogenesis factors.

SUMMARY OF THE INVENTION

Methods are provided for generating nucleic acid ligands to HGF and c-met. The methods use the SELEX process for ligand generation. The nucleic acid ligands provided by the invention are useful as therapeutic and diagnostic agents for a number of diseases.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 illustrates the template and primer oligonucleotides used 2'-F-pyrimidine RNA SELEX experiments. The 5' fixed region of the template and primers contains a T7 promoter to facilitate transcription of RNA by T7 RNA polymerase.

FIGURE 2 illustrates RNaseH cleavage primers used in hybridization truncate SELEX. Bases depicted in bold-type are 2'-O-methyl modified and bases underlined are deoxyribonucleosides. The random region is designated as "N". Upon treatment with RNaseH, the fixed regions are removed at the positions indicated by the carets. Note that the there are two possible cleavage sites at the 5-prime end of the fixed region, resulting in RNA which has one or two fixed G residues.

FIGURE 3 illustrates binding of SELEX pools to HGF. FIGURE 3A shows HGF SELEX 1 30N7 pools. FIGURE 3B shows HGF SELEX 2 30N8 pools.

FIGURE 4 illustrates two methods of evaluating HGF SELEX 3 30N7 pool binding to HGF. In FIGURE 4A, heparin competes with RNA pools for binding to 2.7 nM HGF. FIGURE 4B illustrates conventional pool binding.

FIGURE 5 illustrates two methods of evaluating HGF SELEX 3 30N7 pool binding to HGF. FIGURE 5A shows that tRNA competes with RNA pools for binding to 2.7 nM HGF. FIGURE 5B shows conventional pool binding.

FIGURE 6 illustrates inhibition of 10 ng/ml HGF stimulation of starved HUVECs by aptamers. FIGURE 6A shows a 1st set of aptamers. FIGURE 6B illustrates a 2nd set of aptamers.

FIGURE 7 illustrates truncates of aptamer 8-102. FIGURE 7A shows predicted two-dimensional structures of full-length and truncated sequences. FIGURE 7B shows binding of full-length and truncated aptamers to HGF.

FIGURE 8 illustrates truncates of aptamer 8-17. FIGURE 8A shows a predicted two-dimensional structures of full-length and truncated sequences. FIGURE 8B shows binding of full-length and truncated aptamers to HGF.

FIGURE 9 illustrates binding of HGF truncate SELEX pools. FIGURE 9A shows the HGF SELEX 4 30N7 series. FIGURE 9B shows the HGF SELEX 5 30N7 series.

FIGURE 10 shows aptamer inhibition of 100 ng/ml HGF stimulation of 4MBr-5 cells.

FIGURE 11 illustrates aptamer inhibition of 50 ng/ml HGF stimulation of 4MBr5 cells. FIGURE 11A shows the effect of PEGylation of 36mer. FIGURE 11B shows a comparison of PEGylated 36mer to best full-length inhibitor 8-17.

FIGURE 12 shows aptamer inhibition of 50 ng/ml HGF stimulation of 4MBr-5 cells.

FIGURE 13 shows HUVEC mitogenesis by 10 ng/ml HGF, 10 ng/ml VEGF, or both HGF and VEGF.

FIGURE 14 illustrates aptamer-mediated inhibition of HUVEC mitogenesis. FIGURE 14A shows stimulation by both HGF and VEGF inhibited by either HGF or VEGF aptamers or both. FIGURE 14B illustrates stimulation by HGF alone inhibited by either HGF or VEGF aptamer or both. FIGURE 14C illustrates stimulation by VEGF alone inhibited by either HGF or VEGF aptamer or both.

FIGURE 15 depicts ratios of selected to unselected partially 2'-O-methyl substituted purines in aptamer NX22354.

FIGURE 16 illustrates 2'-O-methyl substituted derivatives of NX22354 binding to HGF: average of two experiments.

FIGURE 17 illustrates binding of SELEX pools to c-met. FIGURE 17A shows c-Met SELEX 40N7. FIGURE 17B shows c-Met SELEX 30N8. FIGURE 17C shows both SELEXes: a, c pools, 40N7; b, d pools, 30N8.

FIGURE 18 illustrates binding of c-met SELEX pools to c-met and KDR Ig fusion proteins.

FIGURE 19 shows binding of c-met 40N7 cloned aptamers to c-met and KDR Ig fusion proteins. FIGURE 19A shows clone 7c-1. FIGURE 19B shows clone7c-3.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The central method utilized herein for identifying nucleic acid ligands to HGF and c-met is called the SELEX process, an acronym for Systematic Evolution of Ligands by Exponential enrichment and involves (a) contacting the candidate mixture of nucleic acids with HGF or c-met, or expressed domains or peptides corresponding to HGF or c-met, (b) partitioning between members of said candidate mixture on the basis of affinity to HGF or c-met, and c) amplifying the selected molecules to yield a mixture of nucleic acids enriched for nucleic acid sequences with a relatively higher affinity for binding to HGF or c-met.

Definitions

Various terms are used herein to refer to aspects of the present invention. To aid in the clarification of the description of the components of this invention, the following definitions are provided:

As used herein, "nucleic acid ligand" is a non-naturally occurring nucleic acid having a desirable action on a target. Nucleic acid ligands are often referred to as "aptamers". The term aptamer is used interchangeably with nucleic acid ligand throughout this application. A desirable action includes, but is not limited to, binding of the target, catalytically changing the target, reacting with the target in a way which modifies/alters the target or the functional activity of the target, covalently attaching to the target as in a suicide inhibitor, facilitating the reaction between the target and another molecule. In the preferred embodiment, the action is specific binding affinity for a target molecule, such target molecule being a three dimensional chemical structure other than a polynucleotide that binds to the nucleic acid ligand through a mechanism which predominantly depends on Watson/Crick base pairing or triple helix binding, wherein the nucleic acid ligand is not a nucleic acid having the known physiological function of being bound by the target molecule. In the present invention, the targets are c-met and HGF or portions thereof. Nucleic acid ligands include nucleic acids that are identified from a candidate mixture of nucleic acids, said nucleic acid ligand being a ligand of a given target, by the method comprising: a) contacting the candidate mixture with the target, wherein nucleic acids having an increased affinity to the target relative to the candidate mixture may be partitioned from the remainder of the candidate mixture; b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and c) amplifying the increased affinity nucleic acids to yield a ligand-enriched mixture of nucleic acids.

As used herein, "candidate mixture" is a mixture of nucleic acids of differing sequence from which to select a desired ligand. The source of a candidate mixture can be from naturally-occurring nucleic acids or fragments thereof, chemically synthesized nucleic acids, enzymatically synthesized nucleic acids or nucleic acids made by a combination of the foregoing techniques. In a preferred embodiment, each nucleic acid has fixed sequences surrounding a randomized region to facilitate the amplification process.

As used herein, "nucleic acid" means either DNA, RNA, single-stranded or double-stranded, and any chemical modifications thereof. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

"SELEX" methodology involves the combination of selection of nucleic acid ligands which interact with a target in a desirable manner, for example binding to a protein, with amplification of those selected nucleic acids. Optional iterative cycling of the selection/amplification steps allows selection of one or a small number of nucleic acids which interact most strongly with the target from a pool which contains a very large number of nucleic acids. Cycling of the selection/amplification procedure is continued until a selected goal is achieved. In the present invention, the SELEX methodology is employed to obtain nucleic acid ligands to HGF and c-met.

The SELEX methodology is described in the SELEX Patent Applications.

"SELEX target" or "target" means any compound or molecule of interest for which a ligand is desired. A target can be a protein, peptide, carbohydrate, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, substrate, metabolite, transition state analog, cofactor, inhibitor, drug, dye, nutrient, growth factor, etc. without limitation. In this application, the SELEX targets are HGF and c-met. In particular, the SELEX targets in this application include purified HGF and c-met, and fragments thereof, and short peptides or expressed protein domains comprising HGF or c-met. Also includes as targets are fusion proteins comprising portions of HGF or c-met and other proteins.

As used herein, "solid support" is defined as any surface to which molecules may be attached through either covalent or non-covalent bonds. This includes, but is not limited to, membranes, microtiter plates, magnetic beads, charged paper, nylon, Langmuir-Bodgett films,

functionalized glass, germanium, silicon, PTFE, polystyrene, gallium arsenide, gold, and silver. Any other material known in the art that is capable of having functional groups such as amino, carboxyl, thiol or hydroxyl incorporated on its surface, is also contemplated. This includes surfaces with any topology, including, but not limited to, spherical surfaces and grooved surfaces.

As used herein, "HGF" refers to hepatocyte growth factor/scatter factor. This includes purified hepatocyte growth factor/scatter factor, fragments of hepatocyte growth factor/scatter factor, chemically synthesized fragments of hepatocyte growth factor/scatter factor, derivatives or mutated versions of hepatocyte growth factor/scatter factor, and fusion proteins comprising hepatocyte growth factor/scatter factor and another protein. "HGF" as used herein also includes hepatocyte growth factor/scatter factor isolated from species other than humans.

As used herein "c-met" refers to the receptor for HGF. This includes purified receptor, fragments of receptor, chemically synthesized fragments of receptor, derivatives or mutated versions of receptor, and fusion proteins comprising the receptor and another protein. "c-met" as used herein also includes the HGF receptor isolated from a species other than humans.

Note that throughout this application, various references are cited. Every reference cited herein is specifically incorporated in its entirety.

A. Preparing nucleic acid ligands to HGF and c-met.

In the preferred embodiment, the nucleic acid ligands of the present invention are derived from the SELEX methodology. The SELEX process is described in United States Patent Application Serial No. 07/536,428, entitled Systematic Evolution of Ligands by Exponential Enrichment, now abandoned, United States Patent No. 5,475,096 entitledNucleic Acid Ligands, and United States Patent No. 5,270,163 (see also WO 91/19813) entitled Methods for Identifying Nucleic Acid Ligands. These applications, each specifically incorporated herein by reference, are collectively called the SELEX Patent Applications.

The SELEX process provides a class of products which are nucleic acid molecules, each having a unique sequence, and each of which has the property of binding specifically to a desired target compound or molecule. Target molecules are preferably proteins, but can also include among others carbohydrates, peptidoglycans and a variety of small molecules. SELEX

methodology can also be used to target biological structures, such as cell surfaces or viruses, through specific interaction with a molecule that is an integral part of that biological structure.

In its most basic form, the SELEX process may be defined by the following series of steps:

1) A candidate mixture of nucleic acids of differing sequence is prepared. The candidate mixture generally includes regions of fixed sequences (i.e., each of the members of the candidate mixture contains the same sequences in the same location) and regions of randomized sequences. The fixed sequence regions are chosen either: (a) to assist in the amplification steps described below, (b) to mimic a sequence known to bind to the target, or (c) to enhance the concentration of a given structural arrangement of the nucleic acids in the candidate mixture. The randomized sequences can be totally randomized (i.e., the probability of finding a base at any position being one in four) or only partially randomized (e.g., the probability of finding a base at any location can be selected at any level between 0 and 100 percent).

2) The candidate mixture is contacted with the selected target under conditions favorable for binding between the target and members of the candidate mixture. Under these circumstances, the interaction between the target and the nucleic acids of the candidate mixture can be considered as forming nucleic acid-target pairs between the target and those nucleic acids having the strongest affinity for the target.

3) The nucleic acids with the highest affinity for the target are partitioned from those nucleic acids with lesser affinity to the target. Because only an extremely small number of sequences (and possibly only one molecule of nucleic acid) corresponding to the highest affinity nucleic acids exist in the candidate mixture, it is generally desirable to set the partitioning criteria so that a significant amount of the nucleic acids in the candidate mixture (approximately 5-50%) are retained during partitioning.

4) Those nucleic acids selected during partitioning as having the relatively higher affinity for the target are then amplified to create a new candidate mixture that is enriched in nucleic acids having a relatively higher affinity for the target.

5) By repeating the partitioning and amplifying steps above, the newly formed candidate mixture contains fewer and fewer unique sequences, and the average degree of

affinity of the nucleic acids to the target will generally increase. Taken to its extreme, the SELEX process will yield a candidate mixture containing one or a small number of unique nucleic acids representing those nucleic acids from the original candidate mixture having the highest affinity to the target molecule.

The basic SELEX method has been modified to achieve a number of specific objectives. For example, United States Patent Application Serial No. 07/960,093, filed October 14, 1992, now abandoned, and United States Patent No. 5,707,796 both entitled "Method for Selecting Nucleic Acids on the Basis of Structure," describe the use of the SELEX process in conjunction with gel electrophoresis to select nucleic acid molecules with specific structural characteristics, such as bent DNA. United States Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands,", now abandoned, United States Patent No. 5,763,177 entitled "Systematic Evolution of Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX" and United States Patent Application Serial No. 09/093,293, filed June 8 1998, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX" all describe a SELEX based method for selecting nucleic acid ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. United States Patent No. 5,580,737 entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," describes a method for identifying highly specific nucleic acid ligands able to discriminate between closely related molecules, termed Counter-SELEX. United States Patent No. 5,567,588 entitled "Systematic Evolution of Ligands by Exponential Enrichment: Solution SELEX," describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule. United States Patent No. 5,496,938 entitled "Nucleic Acid Ligands to HIV-RT and HIV-1 Rev," describes methods for obtaining improved nucleic acid ligands after SELEX has been performed. United States Patent No. 5,705,337 entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chemi-SELEX," describes methods for covalently linking a ligand to its target.

The SELEX method encompasses the identification of high-affinity nucleic acid ligands containing modified nucleotides conferring improved characteristics on the ligand, such as

improved in vivo stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX-identified nucleic acid ligands containing modified nucleotides are described in United States Patent No. 5,660,985 entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. United States Patent No. 5,637,459, supra, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH_2), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2' Modified Nucleosides by Intramolecular Nucleophilic Displacement," now abandoned, describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United States Patent No. 5,637,459 entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX," and United States Patent No. 5,683,867 entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules.

In United States Patent No. 5,496,938 methods are described for obtaining improved nucleic acid ligands after the SELEX process has been performed. This patent, entitled Nucleic Acid Ligands to HIV-RT and HIV-1 Rev, is specifically incorporated herein by reference.

One potential problem encountered in the diagnostic use of nucleic acids is that oligonucleotides in their phosphodiester form may be quickly degraded in body fluids by intracellular and extracellular enzymes such as endonucleases and exonucleases before the desired effect is manifest. Certain chemical modifications of the nucleic acid ligand can be made to increase the in vivo stability of the nucleic acid ligand or to enhance or to mediate the delivery of the nucleic acid ligand. See, e.g., U.S. Patent Application Serial No. 08/117,991, filed September 8, 1993, now abandoned, and United States Patent No. 5,660,985, both entitled

"High Affinity Nucleic Acid Ligands Containing Modified Nucleotides", and the U.S. Patent Application entitled "Transcription-free SELEX", United States Patent Application Serial No. 09/362,578, filed July 28, 1999, each of which is specifically incorporated herein by reference. Modifications of the nucleic acid ligands contemplated in this invention include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrophobicity, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, phosphorothioate or alkyl phosphate modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping. In preferred embodiments of the instant invention, the nucleic acid ligands are RNA molecules that are 2'-fluoro (2'-F) modified on the sugar moiety of pyrimidine residues.

The modifications can be pre- or post-SELEX process modifications. Pre-SELEX process modifications yield nucleic acid ligands with both specificity for their SELEX target and improved in vivo stability. Post-SELEX process modifications made to 2'-OH nucleic acid ligands can result in improved in vivo stability without adversely affecting the binding capacity of the nucleic acid ligand.

Other modifications are known to one of ordinary skill in the art. Such modifications may be made post-SELEX process (modification of previously identified unmodified ligands) or by incorporation into the SELEX process.

The nucleic acid ligands of the invention are prepared through the SELEX methodology that is outlined above and thoroughly enabled in the SELEX applications incorporated herein by reference in their entirety. The SELEX process can be performed using purified HGF or c-met, or fragments thereof as a target. Alternatively, full-length HGF or c-met, or discrete domains of HGF or c-met, can be produced in a suitable expression system. Alternatively, the SELEX process can be performed using as a target a synthetic peptide that includes sequences found in

HGF or c-met. Determination of the precise number of amino acids needed for the optimal nucleic acid ligand is routine experimentation for skilled artisans.

In some embodiments, the nucleic acid ligands become covalently attached to their targets upon irradiation of the nucleic acid ligand with light having a selected wavelength. Methods for obtaining such nucleic acid ligands are detailed in United States Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands,", now abandoned, United States Patent No. 5,763,177 entitled "Systematic Evolution of Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX" and United States Patent Application Serial No. 09/093,293, filed June 8 1998, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX" each of which is specifically incorporated herein by reference in its entirety.

In preferred embodiments, the SELEX process is carried out using HGF or c-met attached to a solid support. A candidate mixture of single stranded RNA molecules is then contacted with the solid support. In especially preferred embodiments, the single stranded RNA molecules have a 2'-fluoro modification on C and U residues, rather than a 2'-OH group. After incubation for a predetermined time at a selected temperature, the solid support is washed to remove unbound candidate nucleic acid ligand. The nucleic acid ligands that bind to the HGF or c-met protein are then released into solution, then reverse transcribed by reverse transcriptase and amplified using the Polymerase Chain Reaction. The amplified candidate mixture is then used to begin the next round of the SELEX process.

In the above embodiments, the solid support can be a nitrocellulose filter. Nucleic acids in the candidate mixture that do not interact with the immobilized HGF or c-met can be removed from this nitrocellulose filter by application of a vacuum. In other embodiments, the HGF or c-met target is adsorbed on a dry nitrocellulose filter, and nucleic acids in the candidate mixture that do not bind to the HGF or c-met are removed by washing in buffer. In other embodiments, the solid support is a microtiter plate comprised of, for example, polystyrene.

In still other embodiments, the HGF or c-met protein is used as a target for Truncate SELEX, described in United States Patent Application Serial No. 09/275,850, filed March 24

1999, entitled "The Truncation SELEX Method", incorporated herein by reference in its entirety.

In preferred embodiments, the nucleic acid ligands thus obtained are assayed for their ability to inhibit the HGF/c-met interaction. In one embodiment, this is performed by performing a cell migration assay. Certain cell types, such as A549 lung carcinoma cells, will show increased migration through a Matrigel-coated filter insert (Becton Dickinson) in the presence of HGF. Thus, the degree of inhibition of HGF activity in the presence of an HGF or c-met nucleic acid ligand can be assayed by determining the number of cells that have migrated through the filter in the presence of HGF.

B. Methods and compositions for using nucleic acid ligands to treat and diagnose disease

Given that elevated levels of c-met and HGF are observed in hypertension, arteriosclerosis, myocardial infarction, and rheumatoid arthritis, nucleic acid ligands will serve as useful therapeutic and diagnostic agents for these diseases. In some embodiments, inhibitory nucleic acid ligands of HGF and c-met are administered, along with a pharmaceutically accepted excipient to an individual suffering from one of these diseases. Modifications of these nucleic acid ligands are made in some embodiments to impart increased stability upon the nucleic acid ligands in the presence of bodily fluids. Such modifications are described and enabled in the SELEX applications cited above.

In other embodiments, nucleic acid ligands to HGF and c-met are used to measure the levels of these proteins in an individual in order to obtain prognostic and diagnostic information. Elevated levels of c-met and HGF are associated with tumors in the liver, breast, pancreas, lung, kidney, bladder, ovary, brain, prostrate, and gallbladder. Elevated levels of HGF and c-met are also associated with myeloma.

In other embodiments, nucleic acid ligands that inhibit the HGF/c-met interaction are used to inhibit tumorigenesis, by inhibiting, for example, angiogenesis and motogenesis.

In one embodiment of the instant invention, a nucleic acid ligand to HGF is used in combination with nucleic acid ligands to VEGF (vascular endothelial growth factor) and/or bFGF (basic fibroblast growth factor) to inhibit tumor metastasis and angiogenesis. The use of multiple nucleic acid ligands is likely to have an additive or synergistic effect on tumor

suppression. Nucleic acid ligands that inhibit VEGF are described in United States Patent No. 5,849, 479, United States Patent No. 5,811,533, and United States Patent Application Serial No. 09/156,824, filed September 18, 1998, each of which is entitled "High Affinity Oligonucleotide Ligands to Vascular Endothelial Growth Factor", and each of which is specifically incorporated herein by reference in its entirety. Nucleic acid ligands to VEGF are also described in United States Patent No. 5,859,228, United States Patent Application Serial No. 08/870,930, filed June 6, 1997, United States Patent Application Serial No. 08/897,351, filed July 21, 1997, and United States Patent Application Serial No. 09/254,968, filed March 16 1999, each of which is entitled "Vascular Endothelial Growth Factor (VEGF) Nucleic Acid Ligand Complexes," and each of which is specifically incorporated by reference in its entirety. Nucleic acid ligands to bFGF are described in United States Patent No. 5,639,868 entitled "High Affinity RNA ligands for Basic Fibroblast Growth Factor", and United States Patent Application Serial No. 08/442,423, filed May 16, 1995, entitled "High Affinity RNA Ligands for Basic Fibroblast Growth Factor", each of which is specifically incorporated herein by reference in its entirety.

EXAMPLES

The following examples are given by way of illustration only. They are not to be taken as limiting the scope of the invention in any way.

Materials and Methods

In the sections below entitled "Results: HGF" and "Results: c-met", the following materials and methods were used:

Proteins. The HGF protein and c-met-IgG_1-His_6 fusion protein, which were used in the SELEX process, and the KDR-IgG_1-His_6 proteins were purchased from R&D Systems, Inc. (Minneapolis, MN). The human c-met-IgG_1-His_6 fusion protein - described from the amino to the carboxyl terminus - consists of 932 amino acids from the extracellular domains of the α and β chains of c-met, a factor Xa cleavage site, 231 amino acids from human IgG_1 (Fc domain), and a $(His)_6$ tag. This protein is referred to in the text and figures as c-met. A similar fusion protein containing the vascular endothelial growth factor receptor KDR will be referred to as KDR.

Anti-HGF monoclonal antibody MAB294 was purchased from R&D Systems, Inc. Human IgG_1 was produced in-house by stable expression from Chinese hamster ovary cells.

SELEX templates and primers. Standard SELEX templates carrying 30 or 40 random nucleotides flanked by fixed regions of the N7 or N8 series and associated primers (FIGURE 1) were used as described (Fitzwater and Polisky 1996, Methods Enzymol. 267:275-301). Truncate SELEX was done by the hybridization method described in United States Patent Application Serial No. 09/275,850, filed March 24 1999, entitled "The Truncation SELEX Method", incorporated herein by reference in its entirety, using RNaseH cleavage primers (FIGURE 2).

SELEX methods. Initial HGF SELEX experiments were done by two closely-related partitioning methods, both involving separating free from bound RNA on nitrocellulose filters. Conventional SELEX involves mixing target protein and RNA library in HBSMC buffer (hepes-buffered saline, 25 mM hepes, 137 mM NaCl, 5 mM KCl plus 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4), followed by filtration on nitrocellulose under vacuum. Maintaining vacuum, the filter is washed in buffer, followed by vacuum release and RNA extraction. In spot filter SELEX, the protein is applied to a dry nitrocellulose 13 mm filter, allowed to adsorb for several minutes, then pre-incubated in Buffer S (HBSMC buffer plus 0.02% each of ficoll, polyvinylpyrrolidone, and human serum albumin) for 10 minutes at 37° C to remove unbound protein. The wash buffer is removed, and then the RNA library is added in the same buffer, and incubated with the protein-bound filter. The filters are washed by repeated incubations in fresh buffer, followed by RNA extraction.

SELEX was initiated with between 1 and 5 nmoles of 2'-fluoro-pyrimidine RNA sequence libraries containing either a 30 or 40 nucleotide randomized region sequence (FIGURE 1). The RNA libraries were transcribed from the corresponding synthetic DNA templates that were generated by Klenow extension (Sambrook, Fritsch *et al.* 1989, 3:B.12). The DNA templates were transcribed in 1 ml reactions, each containing 0.25 nM template, 0.58 μM T7 RNA polymerase, 1 mM each of ATP and GTP, 3 mM each of 2'-F-CTP and 2'-F-UTP, 40 mM Tris-HCl (pH 8.0), 12 mM $MgCl_2$, 1 mM spermidine, 5 mM DTT, 0.002% Triton X-

100 and 4% polyethylene glycol (w/v) for at least 4 hours at 37°C. The full-length transcription products were purified by denaturing polyacrylamide gel electrophoresis. Radiolabeled RNA was obtained from transcription reactions as described above, but containing 0.2 nM ATP and 100 μCi of α-^{32}P-ATP. Alternatively, radiolabeled RNA was obtained by labeling the 5'-end of RNA with α-^{32}P-ATP (NEN-DuPont), catalyzed by T4 polynucleotide kinase (New England Biolabs). To prepare RNA containing 5'-OH groups for kinase reactions, transcription reactions included 5 mM guanosine.

For conventional filter SELEX, radiolabeled RNA pools were suspended in HBSMC buffer to which HGF protein was added, and incubated at 37°C for 30 minutes to 3 hours depending on the round. Binding reactions were then filtered under suction through 0.45 μm nitrocellulose filters (Millipore), pre-wet with binding buffer. The filters were immediately washed with at least 5 ml of HBSMC buffer. For each binding reaction, a protein-minus control reaction was done in parallel in order to determine the amount of background binding to the filters. The amount of RNA retained on the filters was quantified by Cherenkov counting, and compared with the amount input into the reactions. Filter-retained RNA was extracted with phenol and chloroform, and isolated by ethanol precipitation in the presence of 1-2 μg glycogen.

The isolated RNA was subsequently used as a template for avian myeloblastosis virus reverse transcriptase (AMV-RT, Life Sciences) to obtain cDNA. One hundred pmoles of the 3'-primer (FIGURE 1) was added to the RNA and annealed by heating for 3 minutes at 70°C, followed by chilling on ice. The 50 μl reaction contained 5 U AMV-RT, 0.4 mM each of dNTPs, 50 mM Tris-HCl (pH 8.3), 60 mM NaCl, 6 mM $Mg(OAc)_2$, and 10 mM DTT, which was incubated for 45 minutes at 48°C. The cDNA was amplified by PCR with the 5'- and the 3'-primers (FIGURE 1), and the resulting DNA template was transcribed to obtain RNA for the next round of SELEX.

To minimize selection of undesirable nitrocellulose-binding sequences, beginning in round three, we pre-soaked pools with nitrocellulose filters before incubating with the target protein. This treatment worked well to control background binding and helped ensure that each SELEX round had a positive signal/noise ratio. The progress of SELEX was monitored by nitrocellulose filter-binding analysis of the enriched pools (see below).

Truncate SELEX was performed by the hybridization method described in United States Patent Application Serial No. 09/275,850, filed March 24 1999, entitled "The Truncation SELEX Method", incorporated herein by reference in its entirety. Briefly, 2'-F-RNA pools were body-labeled during transcription and cleaved by RNaseH using specific cleavage primers to remove the fixed sequences from the SELEX pool (FIGURE 2). This RNA was then bound to target protein HGF and recovered following partitioning as in a conventional filter SELEX experiment. The recovered RNA was then biotinlyated at its 3-prime end and hybridized overnight under appropriate conditions with single-stranded full-length complementary strand DNA obtained from the starting SELEX pool, from which the RNA had been transcribed. The RNA/DNA complexes were then captured on streptavidin-coated magnetic beads and extensively washed to remove non-hybridized DNA. The bound DNA in the captured RNA/DNA complexes was then eluted by heat denaturation and amplified using conventional SELEX PCR primers. To complete the cycle, the resulting DNA was then used as a transcription template for generating RNA to be cleaved by RNaseH, and used in the next round of truncate SELEX.

For plate SELEX, a polystyrene well was pre-blocked in 400 µl of blocking agent for 60 minutes at 37 °C. The blocking agent was removed and the desired amount of RNA in 100 µl binding buffer was added and incubated for 60 minutes at 37 °C. White, polystyrene breakaway wells (catalog #950-2965) used for partitioning were from VWR (Denver, CO). The blocking agents, I-block and Superblock, were purchased from Tropix (Bedford, MA) and Pierce (Rockford, IL), respectively. The preadsorbtion was done to remove any nucleic acids which might bind to the well or the blocking agent. The random and round one libraries were not preadsorbed to plates to avoid loss of unique sequences. C-met protein was diluted in HBSMCK (50 mM HEPES, pH 7.4, 140 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$), and was adsorbed to polystyrene wells by incubating 100 µl of diluted protein per well for 60 minutes at 37 °C. The wells were each washed with three 400 µl aliquots of HIT buffer (HBSMCK, 0.1% I-block, 0.05 % Tween 20), and then blocked in 400 µl of blocking agent for 60 minutes at 37 °C. SELEX was initiated by incubating 100 µl of RNA in the protein-bound well for 60 minutes at 37 °C. The RNA was removed and the wells were washed with 400 µl aliquots of HIT buffer. Increasing numbers of washes were used in later rounds. The wells

were then washed twice with 400 µl water. RNA bound to c-met was eluted by adding 100 µl water and heating at 95 °C for 5 minutes and then cooled on ice, followed by reverse transcription.

Nitrocellulose filter-binding. In binding reactions, RNA concentrations were kept as low as possible - between 1 and 20 pM - to ensure equilibrium in conditions of protein excess. Oligonucleotides were incubated for 15 minutes at 37°C with varying amounts of the protein in 43 µl of the binding buffer. Thirty-two microliters of each binding mixture placed on pre-wet 0.45 µm nitrocellulose filters under suction. Each well was immediately washed with 0.5 ml binding buffer. The amount of radioactivity retained on the filters was quantitated by imaging. The radioactivity that bound to filters in the absence of protein was used for background correction. The percentage of input oligonucleotide retained on each filter spot was plotted against the corresponding log protein concentration. The nonlinear least square method was used to obtain the dissociation constant (K_d; reference Jellinek, Lynott *et al.* 1993, Proc. Natl. Acad. Sci. USA. 90:11227-31).

Competitor titration curves were generated essentially as a standard binding curve, except that the protein and RNA concentrations were kept constant, and the competitor concentration was varied. Competitors were also added at a fixed concentration in binding experiments to increase stringency for purposes of comparing pool binding affinities. In these experiments, the competitor concentration was chosen based on the results from the competitor titration curves.

Molecular cloning and DNA sequencing. To obtain individual sequences from the enriched pools, we cloned the PCR products from the final SELEX rounds using one of two blunt-end cloning kits, Perfectly Blunt (Novagen, Madison, WI), or PCR-Script (Stratagene, La Jolla, CA). Clones were sequenced with the ABI Prism Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, CA). Sequences were obtained from an automated ABI sequencer, and text files were collated and analyzed by computer alignment and inspection.

Boundary determinations. Five-prime and 3-prime boundaries of RNA aptamers were determined by the method of partial alkaline hydrolysis as described (Jellinek, Green *et al.* 1994, Biochemistry. 33:10450-6).

Cell assays. Standard cell culture procedures were employed in the course of performing *in vitro* experiments to test aptamer-mediated inhibition of HGF activity. For cell migration assays, monolayers of A549 (lung carcinoma) cells were grown on the top-sides of Matrigel-coated filter inserts (Becton Dickinson, Franklin Lakes, NJ) in 24-well plates. The cells adhere to the upper surface of the filter, which is placed in growth medium containing HGF. After two days, the cells are physically removed from the top surface of the filter. The filter is then removed from the insert and stained with crystal violet. Since all cells on the top of the filter are gone, the only cells that remain are those that are have migrated to the bottom of the filter. In the presence of HGF, significantly more cells are found on the bottom of the filter compared to controls without HGF.

Oligonucleotide synthesis and modification. RNA was routinely synthesized by standard cyanoethyl chemistry as modified (Green, Jellinek *et al.* 1995, Chem Biol. 2:683-95). Two-prime-fluoro-pyrimidine phosphoramidite monomers were obtained from JBL Scientific (San Luis Obispo, CA); 2'-OMe purine, 2'-OH purine, hexyl amine, and the dT polystyrene solid support were obtained from Glen Research (Sterling, VA).

For addition of 40K-PEG, RNA oligomers were synthesized with an amino-linker at the 5'-position. This was subsequently reacted with NHS-ester 40K-PEG manufactured by Shearwater Polymers, Inc. (Huntsville, AL), and purified by HPLC on a reverse-phase preparative column.

2'-O-methyl purine substitution. Determination of which 2'-OH-purines can be substituted by 2'-O-methyl-purine was done as described (Green 1995, Chem Biol. 2:683-95). Briefly, a set of oligonucleotides was synthesized with a mixture of 2'-O-methyl amidites and 2'-OH amidites at defined purine positions. The set was designed so that each oligonucleotide contains a subset of partially-substituted purines, and the complete set encompasses all purines. Each aptamer

was 5'-end labeled and subjected to limited alkaline hydrolysis followed by binding to HGF protein at two different concentrations, 50 and 100 pM. Following binding, protein-bound RNA was separated by standard nitrocellulose filtration. Bound RNA was recovered and analyzed by high-resolution gel electrophoresis. The fragmented alkaline-hydrolyzed aptamers which were not exposed to HGF were run to establish the cleavage patterns of the unselected aptamers. Hydrolysis occurs only at 2'-OH-purines. If a given position requires 2'-OH for optimal binding to HGF, it appears as a relatively darker band compared to the unselected aptamer at that position.

Results - HGF

Five HGF SELEX experiments were done in total. The first three were done by conventional filter SELEX, while the latter two were done by the hybridization truncate SELEX method described in United States Patent Application Serial No. 09/275,850, filed March 24 1999, entitled "The Truncation SELEX Method", incorporated herein by reference in its entirety. HGF SELEX 1 was done with 30N7 2'-F-RNA for thirteen rounds of conventional filter binding. HGF SELEX 2 was done with 30N8 2'-F-RNA for thirteen rounds of conventional filter binding. HGF SELEX 3 was done with 30N7 2'-F-RNA for seven rounds by spot filter binding, followed by eight rounds of filter binding. HGF SELEX 4 was done by hybridization filter SELEX for three rounds, starting with pool 8 from HGF SELEX 1. HGF SELEX 5 was done by hybridization filter SELEX for three rounds, starting with pool 11 from HGF SELEX 3. HBSMC buffer was used in conventional SELEX reactions, and in spot filter SELEX, blocking agents were added as described in Materials and Methods.

RNA pool binding with and without competitors heparin and tRNA. To evaluate SELEX progress, binding curves with purified HGF protein were routinely done with evolved pools during the course of these experiments. Representative binding curves are shown for HGF SELEX experiments 1 and 2 (FIGURE 3). These data were used to ascertain when a SELEX was complete in that further progress was not likely to occur by performing additional rounds. HGF SELEX 1 reached its maximal binding by round 8, with a binding affinity of approximately 0.1 nM (FIGURE 3A; earlier rounds and round 9 were examined in other

experiments). HGF SELEX 2 reached its maximal binding by round 10, with a binding affinity of approximately 0.1 nM (FIGURE 3B). HGF SELEX 3 reached its maximal binding by round 11, after seven rounds of spot filter partitioning followed by four rounds of conventional filter SELEX (see FIGURE 4B). A SELEX experiment which was deemed complete was characterized by cloning and sequencing (see below).

HGF, like other proteins which have large clusters of positively charged amino acids, exhibits a high degree of non-specific binding to polyanionic compounds. For example, random RNA pools bind to HGF with low nanomolar affinity, similar to the value reported for HGF binding to heparin, a polyanionic sulfated polysaccharide known to have an important biological role in HGF function (Zioncheck, Richardson *et al.* 1995, J Biol Chem. 270:16871-8). Competition binding to heparin as well as the non-specific competitor tRNA was done to provide an additional means of evaluating SELEX progress. We did this because the binding of random and evolved RNA pools to HGF occurs in a high-affinity range which makes it difficult to monitor progress. In other words, random RNA binds so well to HGF that the affinity enhancement of the evolved pools may not be adequately assessed in conventional binding experiments in the absence of competitor.

RNA pools from HGF SELEX 3 were subjected to competition with heparin (FIGURE 4A). This experiment demonstrates that random RNA is considerably more sensitive to competition for binding to HGF than are the evolved pools. These data are compared to those obtained from a binding curve with the same three RNA pools (FIGURE 4B). In the absence of heparin competition, binding of random RNA to HGF is nearly as good as that of the evolved pools, whereas the heparin competition reveals that the evolved pools are significantly different in composition from random RNA. In addition, while rounds 8 and 11 are indistinguishable in conventional binding curves, round 11 exhibits improved binding based on increased resistance to heparin competition. These data contributed to the choice of round 11 as the maximally binding pool from which we cloned and sequenced.

A similar, but more pronounced, effect was observed with tRNA as the competitor (FIGURE 5A). These data indicate that the round 11 pool from HGF SELEX 3 are at least four orders of magnitude more resistant to competition for binding to HGF than is random RNA. From these curves, it was determined that 800 nM tRNA is the maximum concentration at

which complete binding of evolved RNA persists. Therefore, binding curves were done at this tRNA concentration to compare the binding of different evolved pools (FIGURE 5B). These curves were useful in determining that further SELEX rounds beyond round 11 did not improve binding.

Typical data from a similar set of binding competition experiments done for latter rounds of HGF SELEX 1 are summarized in Table 1.

Cloning and sequence analysis of HGF SELEXes 1, 2 and 3. Following determination of pool binding affinities for HGF, we subjected the optimal SELEX pools to cloning and sequencing in order to isolate and characterize individual aptamers. Data from 30N7 HGF SELEXes 1 and 3 are summarized in Table 2, including binding affinities for many of the aptamers. A similar data set was generated for 30N8 HGF SELEX 2 (Table 3). Sequences from HGF SELEX 1, 2 and 3 are designated 8-seq. number, 10-seq. number, and 11-seq. number, respectively, referring to the total number of SELEX rounds each cloned pool was subjected to. Sequences were analyzed and organized into groups with significant homology. Motifs were analyzed and predicted structures were drawn in order to analyze key features responsible for binding to HGF.

Inhibition of HGF-mediated stimulation of cell proliferation. HGF, while not a potent mitogen, does stimulate moderate proliferation of many cell lines, which can be measured by incorporation of ^{3}H-thymidine. We assayed the inhibitory activity of HGF aptamers by measuring their effect on proliferation of human umbilical vein endothelial cells (HUVECs), or monkey bronchial epithelial (4MBr-5) cells. Based on the binding data and sequence family analysis, fourteen aptamers were chosen for analysis *in vitro* because they bind to HGF with high affinity and are representative of different sequence families. The sequences are shown in Table 4 aligned by a rough consensus which contains bases in common to several families. All sequences are 30N7 except 10-2 which is 30N8.

HGF stimulates proliferation of HUVECs by about two-to-three-fold (data not shown). The initial experiment indicated that aptamers 8-17, 8-102, 8-104, 8-122, 8-126, 10-2 and 11-208 were effective inhibitors of HGF-induced HUVEC proliferation with K_i values in the low

nanomolar range (FIGURE 6). Aptamers 8-113 and 11-222 were less effective and 8-151 exhibited little or no concentration-dependent inhibition. The latter observation is consistent with the fact that aptamer 8-151 does not bind HGF with high affinity and actually binds worse than the random pool.

Several approaches were taken to reduce the length of aptamers which retained significant inhibition of HGF: 1) boundary determinations by biochemical separation of partially hydrolyzed aptamers; 2) sequence motif analysis and educated guessing; and 3) truncate SELEX.

Boundaries and truncation. Boundary determinations were done for a subset of aptamers that demonstrated *in vitro* inhibition of HGF activity. Using a standard alkaline hydrolysis procedure with 5'-end-labeled RNA, we examined the 3'-boundaries of 8-17, 8-102, 8-104, 8-126, 10-1, and 10-2. Additionally, 3'-end-labeled RNA was used for 5'-boundary experiments with 8-17 and 8-102. These experiments were mostly uninformative, probably because the high degree of non-specific binding of RNA fragments, regardless of size, obscured the binding of truncated high-affinity aptamers to HGF. Non-specific binding of virtually all fragments gave no boundary information, and reducing the protein concentration did not help. Instead, we tried to use polyanionic competitors tRNA and heparin to eliminate nonspecific binding to reveal the actual boundaries. The competitors reduced non-specific binding, and HGF was predominantly bound only by full-length aptamers, revealing no boundary information beyond the possibility that full-length aptamers are strongly preferred.

The sole exception was aptamer 8-102 which had a plausible 3'-boundary between two possible endpoints which made sense with respect to computer-predicted structures (FIGURE 7A). Based on the boundary data and structural data, two truncates of 8-102 were synthesized and analyzed for binding to HGF. The sequence of the full-length aptamer and the two truncates are shown, with fixed regions underlined:

gggaggacgaugcggcgagugccuguuuaugucaucguccgucgucagacgacucgcccga 8-102 SEQ ID NO:12

ggacgaugcggcgagugccuguuuaugucaucgucc (36mer) SEQ ID NO:13

gacgaugcggcgagugccuguuuauguc (28mer) SEQ ID NO:14

In binding to HGF, the 36mer bound almost as well as the full-length aptamer, while the 28mer bound no better than random 30N7 (FIGURE 7B), suggesting that the boundary data were correct.

Truncation by sequence structure prediction. Several attempts were made to base truncation on motif analysis and predicted structures, but these did not succeed in producing truncates which retained binding to HGF. For example, aptamer 8-17 folded into a reasonable predicted structure which suggested two obvious points of truncation from its 3-prime terminus, into a 38mer or 28mer (FIGURE 8A). However, binding analysis revealed that neither of these truncates retained significant binding to HGF (FIGURE 8B). These data suggest either that the predicted structure is incorrect or that some of the 3-prime region past base 38 is critical for high-affinity binding of aptamer 8-17 to HGF. These two hypotheses are not mutually exclusive. Nevertheless, we did not succeed in obtaining a useful truncate of 8-17 by boundary and structural prediction.

Truncate SELEX. In order to generate additional short aptamers, we subjected advanced rounds of the earlier SELEXes to additional rounds of truncate SELEX, using the Truncation SELEX method described in United States Patent Application Serial No. 09/275,850, filed March 24 1999, entitled "The Truncation SELEX Method", incorporated herein by reference in its entirety. Binding of RNaseH cleaved pools was examined to determine which were the appropriate rounds to use to initiate truncate SELEX (data not shown). None of the RNaseH-cleaved evolved pools was clearly superior to another in binding to HGF, therefore, the pools which had been previously cloned were chosen to use in truncate SELEX. The encouraging result from this experiment was that after RNaseH treatment, the evolved pools bound better to HGF than did random RNA, suggesting that even in the absence of the fixed regions, significant binding affinity was retained. This observation was sufficient evidence to suggest that truncate SELEX could enrich for sequences which bound to HGF in the absence of fixed regions.

Three rounds of hybridization truncate SELEX were done in parallel, using as starting pools HGF SELEX 1 round 8 and HGF SELEX 3 round 11. The truncate SELEX rounds were done at equi-molar RNA and protein, starting at 1 nM and decreasing to 0.5 and 0.1 nM. Signal-to-noise ratios were very high during selection. Subsequent manipulations were satisfactory even though the amount or recovered RNA was sub-picomolar.

To evaluate the progress of the SELEX, binding affinities of truncate rounds two and three were determined compared to those of the RNaseH-cleaved starting pools (FIGURE 9). For both SELEXes, the third round pools bound with improved affinity for HGF compared with the earlier rounds. Interestingly, the second rounds did not bind HGF better than the staring material. The dissociation constants for the third round truncate SELEX pools are 1-2 nM, representing a 2-3 fold improvement. While the magnitude of this improvement is not large, it is probably significant since HGF as a target did not easily yield affinity enrichment, probably because of its intrinsically high affinity for RNA.

The two pools were cloned and sequenced, and binding affinities were determined (Table 5). The truncated aptamer with the best binding affinity, Tr51, is among several sequences which are novel, that is, they were not found in the clones sequenced from the full-length SELEX pools. The emergence of novel sequences suggests that the truncate SELEX succeeded in amplifying aptamers which were relatively rare in the full-length pools. Aptamer Tr51 appeared more frequently than any other sequence, consistent with the observation that it has better binding affinity than any other truncate. Other sequences which appeared multiple times also tend to be those with binding affinities near or better than the pool K_d of 1-2 nM.

HGF inhibition by the 36mer aptamer modified with 40K-PEG. The 36mer derivative of aptamer 8-102 described above was tested for inhibition *in vitro* in a 4MBr-5 cell proliferation assay (FIGURE 10). Although the 36mer retained high-affinity binding to HGF, it did not retain inhibitory activity *in vitro* comparable to its parent aptamer 8-102 and aptamer 8-17 (FIGURE 10).

In order to improve the activity of the 36mer, we tested it in a formulation with a 3'-dT cap and 5'-40K PEG. The modified aptamer, designated NX22354, was tested for inhibition of HGF-mediated proliferation 4MBr-5 cells (FIGURE 11A). The data indicate that the 36mer-

PEG aptamer inhibits HGF, and that it performs at least as well as the full-length aptamer 8-17, which had previously exhibited the strongest inhibition of all aptamers tested. As expected, the non-PEGylated 36mer did not inhibit HGF, suggesting that the addition of PEG and/or the 3'-cap contribute to the aptamer's bioactivity. This experiment was also done at lower aptamer concentrations, supporting the previous result and showing more clearly that 36mer-PEG aptamer is a better inhibitor that the 8-17 full-length aptamer (FIGURE 11B). Also tested by this assay was a non-binding aptamer containing a 3'-dT cap and 5'-40K PEG, the VEGF aptamer NX1838, which had no effect on HGF stimulation (FIGURE 12). In this same experiment, a non-PEGylated version of NX1838 and the truncate SELEX aptamer Tr51 were shown to have no inhibitory effect on HGF (FIGURE 12). This suggests that Tr51, similar to the 36mer base aptamer of NX22354, may require 5'-40K-PEG to inhibit HGF function.

Inhibition of HGF-mediated stimulation of cell migration. HGF readily stimulates cell movement, hence the name, scatter factor. We assayed the inhibitory effect of HGF aptamers by measuring their effect on A549 cell migration across a Matrigel coated membrane with 8.0 micron pores as described in Materials and Methods (Table 6). The NX22354 aptamer fully inhibited HGF-mediated migration at both 1 and 0.2 μM concentrations, but at 0.04 μM, the effect was negligible. The monoclonal antibody control (sample 3) was moderately effective at the 1 μg/ml dose, which is above its published EC_{50} value of 0.1-0.3 μg/ml for inhibition of 4MBr-5 cell proliferation.

Combined inhibitory effect of HGF and VEGF aptamers on HUVEC proliferation. It was reported that VEGF and HGF have an additive stimulatory effect on HUVEC proliferation (Van Belle 1998, Circulation. 97:381-90). We observed this effect when VEGF and HGF were added, singly and in combination, to HUVECs, and we measured incorporation of ^{3}H-thymidine (FIGURE 13). As expected, stimulation by HGF was relatively weak compared with that of VEGF and together, the stimulatory effect was greater than that elicited by VEGF alone.

Based on these curves, we chose to add each cytokine at 10 ng/ml for optimal stimulation in the aptamer inhibition experiments. We then tested the effect of adding one or both aptamers to the doubly-stimulated cells in the presence of both growth factors (FIGURE 14A). We

observed that each aptamer partially inhibits the stimulation and that both aptamers result in complete inhibition. Interestingly, the magnitude of the inhibitory effect of each aptamer roughly corresponds with the magnitude of the stimulation conferred by each cytokine. This observation suggests that the stimulatory effect of each cytokine can be inhibited independently, and that the two cytokines stimulate HUVECs independently.

The remaining two panels of FIGURE 14 (Fig. 14B and Fig. 14C) are controls in which each cytokine being administered separately, demonstrating that the HGF and VEGF aptamers do not cross-react, that is, each aptamer affects only the cytokine against which it was selected. For the HGF stimulated cells, we observed inhibition by the HGF aptamer NX22354, but not by the VEGF aptamer NX1838 (FIGURE 14B). Conversely, stimulation by VEGF was inhibited by the VEGF aptamer NX1838, but was unaffected by the HGF aptamer NX22354 (FIGURE 14C).

These data, along with the fact that HGF, like VEGF, is an angiogenesis factor make it intriguing to consider dual administration of VEGF and HGF aptamers to treat tumors. Furthermore, the availability of aptamers which inhibit other growth factors suggests further combinations of the VEGF or the HGF aptamer in combination with other aptamers, for example, aptamers that inhibit bFGF, platelet-derived growth factor (PDGF), transforming growth factor beta (TGF), keratinocyte growth factor (KGF), and/or their receptors allowing for the possibility that any combination of these inhibitors may be relevant. The goal is to have an array of aptamer-inhibitors of cytokines and their receptors and to be able to tailor combination treatments for specific disease states.

2'-O-methyl-purine substitution of HGF aptamer NX22354. To improve the stability and pharmacokinetics of NX22354, we determined which of the 17 2'-OH purines could be replaced. We did this by synthesizing four partially substituted 2'-O-methyl-purine variants of the base sequence of NX22354 followed by analysis as described in Materials and Methods. The four partially-substituted oligonucleotides were synthesized with a 1:1 ratio of 2'-O-methyl amidite:2'-OH amidite (Table 7). The data analysis measures the ratios of the selected to unselected RNA at each substituted purine position, based on quantitation of bands from the gel. The data are summarized by position (FIGURE 15). At each position, the three

unsubstituted aptamers provide an important comparison, which is expressed as an average of the three unsubstituted aptamers with standard deviation represented by the error bars. Points that occur at ratios higher than that of the nearby positions are likely to require 2'-OH for binding.

The data strongly indicate that two positions, G5 and A25, do not tolerate 2'-OMe substitution. Two other positions, A3 and G10, show a slight preference above the standard deviation of the unselected RNA.

The set of OMe aptamers were also examined for binding to HGF (data not shown). The binding data indicate that the OMe1 and OMe3 bind as well as the parent unsubstituted 36mer, whereas OMe2 and OMe4 bind less well. This suggests that the substitutions in OMe2 and OMe4 are less well tolerated with respect to HGF binding in solution, consistent with the fact that OMe2 and OMe4 are substituted at A25 and G5, respectively.

To confirm these results, two aptamers were synthesized which are fully 2'-O-methyl substituted at the apparently well-tolerated positions. The sequences are shown below, with the 2'-OH-purines shown underlined. All other purines have 2'-OMe and the pyrimidines are 2'-fluoro substituted.

4x Sub 2'-OH. GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCC
SEQ ID NO:186

2x Sub 2'-OH. GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCC
SEQ ID NO:187

Sequence 4x Sub 2'-OH contains all four of the 2'-OH-purines in question, while 2x Sub 2'-OH has only the two 2'-OH-purines most likely to be required.

Binding of these oligomers to HGF was examined compared to the unsubstituted parent and the fully 2'-O-methyl substituted RNA (FIGURE 16). Based on these binding curves, NX22354 tolerates 2'-OMe substitution at all purines except G5 and A25 (aptamer 2x Sub 2'-OH)with minimal loss of binding affinity. The other two positions in question apparently are

not required to be 2'-OH since aptamer 4x Sub 2'-OH binds no better than aptamer 2x Sub 2'-OH.

Two aptamers have been synthesized with 5'-40K-PEG and a 3'-dT cap: one is fully 2'-O-methyl substituted and the other contains 2'-OH at positions G5 and A25. One of these will presumably supplant NX22354 as the lead HGF aptamer for further testing *in vitro* and *in vivo*.

Results - c-met

c-Met SELEX. In the c-Met plate SELEX experiments, the concentration of nucleic acids was lowered initially, but then raised in later rounds so that the ratio of the nucleic acid to protein would be very high. This was done in order to create conditions of high stringency which may select for higher affinity aptamers. Stringency was also applied by increasing the number of washes.

SELEX pool binding. Binding of SELEX pools to c-met was assessed through round 7 (FIGURE 17). The binding data indicate that the SELEX resulted in about a 20 fold improvement in K_d from 20 nM to 1 nM for both "a" (40N7) and "b" (30N8) pools.

Since the c-met protein used in SELEX is an IgG fusion protein, we tested random 40N7 and round 7c RNA pools for binding to human IgG_1 and c-met. The binding dissociation constants obtained are as follows:

SELEX round	Protein	K_d
random	IgG_1	~1μM
7c	IgG_1	23 nM
random	c-met	100 nM
7c	c-met	2 nM

Table 8: binding and dissociation constants

The affinity of round 7c RNA for both IgG_1 and c-met proteins improved about 50-fold. There are several interpretations to this result. Aptamers may have been selected which

bind with better affinity to both proteins. This assumes that the difference in binding between IgG_1 and c-met is due to c-met specific aptamers. However, the two proteins were made in different cell lines which may have different glycosylation patterns which could influence binding. Thus, if the differences in affinity are due to differences between the free IgG_1 protein and the IgG_1 domain in c-met, then there might be few if any c-met specific aptamers in the round 7 pool.

In order to address these issues further, random and round 5 RNA pools from both libraries were examined for binding to the c-met and KDR proteins (FIGURE 18). Both of these proteins were made in the same cell line and contain the same IgG_1-His_6 sequence. Random RNA from both libraries binds about the same to each protein (K_d = ~50 nM). Round 5 from the both libraries of c-met SELEX binds better to c-met than to KDR (~100-fold better for the 30N8 pool and 3-fold better for the 40N7 pool). However, round 5 RNA pools do bind better than random RNA to KDR. These results imply that, while there are probably aptamers which bind to human IgG_1 or $(HIS)_6$ tag in the round 5 pools, there may also be c-met aptamers.

Detection of IgG aptamers by PCR. Another approach for determining if IgG_1 aptamers are present in the SELEX pools was to subject them to PCR. Predominant IgG_1 aptamers have been isolated from N7 type libraries which have a known sequence (Nikos Pagratis and Chinh Dang, personal communication). For the PCR, a DNA oligonucleotide:

ML-124; 5'-ACGAGTTTATCGAAAAAGAACGATGGTTCCAATGGAGCA-3'
SEQ ID NO:188

was used that is complementary to the most prevalent N7-series human IgG_1 aptamer sequence, and differs by only a few bases from most other IgG_1 aptamers. This PCR primer is the same length as the selected sequence of the major IgG_1 so that it can tolerate mismatches and hybridize to similar sequences.

The ML-124 3'-primer:

ML-34; 5'-CGCAGGATCCTAATACGACTCACTATA-3'
SEQ ID NO:189

was used with a 5'-primer containing the T7-promoter sequence present in all cloned aptamers to amplify 40N7 series nucleic acids pools: random, 1a, 2a, 3a and 4a (data not shown). Since IgG_1 aptamers have not been isolated from an N8 type library, this analysis was not done for the 30N8 SELEX. PCR of random and c-met SELEX round 1a pools yielded no signal after 20 cycles. However, rounds 2a, 3a, and 4a had steadily increasing signals that were easily detectable after 10 PCR cycles. Thus IgG_1 aptamers appeared relatively early in the 40N7 SELEX experiment. For a negative control, PCR was done with a nucleic acid pool from a SELEX known to lack IgG_1 aptamers. For positive controls, PCR was done with pools from either an N7-based IgG_1 or CTLA4-IgG_1 SELEX. IgG_1 aptamers were first isolated from both of these SELEXes. The negative control had no detectable IgG_1 aptamers after 20 PCR cycles. The positive controls had detectable signals after 10 PCR cycles.

C-met aptamers. The sequences of 19 clones from round 7c-40N7 fall into five families with two sequences each, a group with three unrelated members, and six sequences closely related to known IgG_1 aptamer sequences (Table 9). Thus, at least 6 of the 19 clones (32 %) are human IgG_1 aptamers. This confirms the results of previous analysis that indicated the presence of IgG_1 aptamers in this SELEX experiment.

Of the 13 clones sequenced from round 7b-30N8, six are almost identical, another five are closely related, and two are distinct (Table 10).

Nine clones were tested for binding to c-met or KDR, six from the 40N7 series and three from the 30N8 series. These clones were chosen for the following reasons. Clone 7b-4 is the most frequent clone in family 1 and is representative of almost all of the sequences isolated from the 7b-30N8 library. Clones 7b-10 and 7b-12 are the two clones from the 7b-30N8 library that had different sequences. From the 7c-40N7 pool, the chosen representatives were: family 1 (clone 7c-1), family 2 (clone 7c-4), family 3 (clone 7c-23), family 4 (clone 7c-26), family 5 (clone 7c-25), and the presumed IgG1 family (clone 7c-3).

Results are shown for only two clones, including 7c-1 which was the only one observed to bind to c-met better than KDR (FIGURE 19A). Clone 7c-1, which appeared twice in the 40N7 series, may exhibit biphasic binding behavior with a high affinity binding

K_d of ~50 pM and a lower affinity binding K_d of ~5 nM. All eight other clones bound to KDR as well as to c-met, including 7c-3, which is shown here as representative example (FIGURE 19B). Clone 7c-3 and all others besides 7c-1 are presumed to be IgG_1 aptamers.

In summary, two clones (identical to 7c-1) out of 32 apparently bind c-met specifically and with high affinity. The remaining clones appear to be IgG_1 aptamers.

Table 1. Binding affinities of HGF SELEX 1 pools with and without competitor tRNA.

RNA pool	K_d (nM)	K_d (nM) w/ tRNA
random 30N7	1.6	550
HGF SELEX 1 Rd.8	0.07	0.35
HGF SELEX 1 Rd.9	0.09	0.42

Table 2. HGF 30N7 aptamer sequences and binding affinities.

Seq. no.[a]	30N7 random region[b]	SEQ. ID. No.	K_d (nM)
8-122 (2,1)	CGGUGUGAACCUGUUUAUGUCCGCGUACCC	18	0.097
8-108	CGGUGUGGACCUGUUUAUGUCCGCGUACCC	19	ND[c]
8-115	AGUGAUCCUAUUUAUGACAUCGCGGGCUGC	20	ND
8-125	UGUGAACCUGUUUAUGUCAUCUUUUGUCGU	21	0.075
8-155 (1,1)	UGUGAACCUAUUUAUGCCAUCUCGAGUGCC	22	0.093
8-162	CGUGAGCCUAUUUAUGUCAUCAUGUCUGUC	23	ND
8-165	CGAGAGCCUAUUUAUGUCAUCAUGCCUGUG	24	0.100
8-171	CGGGAGCCUUUUUAUGUCAUCAUGUCUGUG	25	0.120
8-114 (4,2)	CGUGAGCCUAUUUAUGUCAUCAUGUCUGUG	26	0.071
8-203	CGCGAGCCUAUUUAUGUCAUCAUGUCUGUG	27	0.140
8-215	CGUGAGCCUAUUUAUGUCAUCAUGUCUGGU	28	0.077
8-217	CGUGAGCCUAUUUACGUCAUCAUGUCUGUG	29	ND
8-222	UGUGAACCUAUUUAUGCCAUUAUGUCUGUG	30	0.130
8-225	CGUGAGCCUAUUUAUGUCAUCAAGUCUGUG	31	ND
8-102	CGAGUGCCUGUUUAUGUCAUCGUCCGUCGU	12	0.060
11-9	CGUGAGCCUGUUUAUGACCUCGUCCAUGGC	32	0.074
11-58	CGUGAGCCUAUUUAUGACAUGUCCCUCGAG	33	ND
11-59	CGUGAGCCUGUAUAUGUCAUUGUUCUCCGG	34	0.110
11-57	UGAGUACCUGUUUAUGUCACCACUUUCCCC	35	ND
11-103	UGAUUACCUA UUAUGUC UCGCCCUCUC	36	0.200
11-110	UGAUUACCUAUUUAUGUCAUGCUCCUCCCC	37	0.086
11-65	UGAUAACCUGUUUAUGCCAUCGUGCUGGGC	38	0.110
11-167	UGAUAACCUGUUUAUGUCAUCGUGCUGGGC	39	ND
11-201	UGAGAACCUAUUUAUGUCAUCGUGUCUGGC	40	ND
11-162	UGAUAACCUAUUUAUGACGUCGUGGCUCCC	41	ND
11-202	UGGGAACCUAUUUAUGUCAUC UCCGUCCC	42	ND
11-106	CGAUGAUGCCUGUUUAUGUCGAUGUCCCCC	43	0.120
11-158	CGAUAGCCUAUUUAUGACCUCGUCCCCGUG	44	0.170
11-112	CGUGAGCCUAUUUAUGACAUCGUUCUUGGC	45	ND
11-124	CGUGAGCCUAUCUAUGUCAUCGUGUGUGCC	46	ND
11-122	UGAGUACUAUUUAUGUCGUCGUUCGUGCC	47	ND
11-217	CGUGAGCCUUCCAAUGACGUCGUCCUUGGC	48	0.071
8-104	GCGACUCAAUCUGAAUCGUCUUGUCCCGUG	49	0.050
11-76	UCAGCGGCGCGAGCCUGUUUAUGUC UGCUG	50	0.076
"consensus"	CGUGAGCCUAUUUAUGUCAUCGU-C-UG	51	
11-8	UCAGUAUGACU UUUAUAGCA CGUUCGCCC	52	0.150
11-153	ACAGGUAGUCU UCUAUAGCA CUUCCUCCCC	53	0.190
11-157	UCAGAAUGACU UUCAUAGCA CGCUUUCCC	54	0.260
11-222	ACAUAAGUCU UCUAUAGC UCGUCCUUUGUG	55	0.077
11-223	UCAGUAUGGCU UCUAUAGC UCGUUCCUCGG	56	0.120
8-126 (3,1)	GUGACUCAAAAUGGUGAUCCUCG UUUCCGC	57	0.099
8-101	GUGACUCAAAAUGGUGAUCCUCGAUUUCCGC	58	0.095
8-105	GUGACUCAAAAUGGUGAUCCUCGAUUGCCGC	59	ND
8-103	GCCGAAAAU UCGUCGACAUCUCCCUGUCUG	60	0.120
8-118	GGCGACUUUCCUCCAAUUCUCACCUCUGCA	61	0.160

Table 2. (cont.)

Seq. no.[a]	30N7 random region[b]	SEQ. ID. No.	K_d (nM)
8-119	GCCAUUCGAUCGA UUCUCCGCCGGAUCGUG	62	0.110
"consensus"	CGUGAGCCUAUUUAUGUCAUCGU-C-UG	51	

8-3 (2)	AUCCCGCGAC CAGGGCGUU UCUUCCUCGUCC	63	0.130
8-112 (3)	UCCCGAAUUUAAGUGCGUU UCCUCCGCGUC	64	0.130
8-154 (3)	UCCCAAGAUUCAGGGCGUU UCUUCCUCGUC	65	0.120
8-117	UCCCAAGUUUCAGGGCGUU UCUUCCUCGUC	15	0.130
8-123	UCCCGAGUUUGAGGGCGUU UCUUCUUCGUC	66	0.210
11-121	UCCCAGUUUCAgGGGCGAU UCCUCUUCGUC	67	ND[c]
8-17 (7,1)	GCGGCU CGAUG UCGUCUUAUCCCUUUGCCC	68	0.095
8-16	GCGGGCU CGAUG UCGUCUUAUCCCCUUUGCCCC	69	ND
8-158	CCGGCU CGAUG UCGUCUUA CCCCUUUGCCC	70	0.310
11-104	GUUUGAG UGAUG UCGUCUUGUCCCGCCUGC	71	0.091
11-111	GUUAGAG UUUUG UCGUCUUGUCCCAUGUG	72	ND
11-163	GCUUGAGUC UUUG AUCGUCUUAUCCCUCGU	73	0.082
11-208	GUUUGAG UGACG AUCGUCUUGUCCCAUGUG	74	0.060
11-212	GUUUGAG UUAAA CAUCGGUUUUCUCCUG	75	0.075
11-6	GACGCG UUGAUU CAUCGUCUUAUCCUGCUG	76	0.240
11-126	GUUUGGGUCU UGAUC UCGUCUUGUCCCGUG	77	0.170
11-165	gUUGAUAGG AGUCAU CAUCGUCUUGUCCGC	78	0.073
11-215	GUAGUGAG UUUUCAUU GUCUUGUCCCCGUG	79	0.091
11-151	UGAGUCAUAGUGUUG AUCGUCGUAUCCCGU	80	0.170
11-7	GUGGAGUCAA AUCGUCUUGUCCCUUGUCCU	81	0.110
11-166	GUUUGAG UUCUGACA CGUCUUGUCCCAUGC	82	0.079
11-17	GUUAGAGC GUGACAG UCGUCUUAUCCCGGGUCA	83	0.130
8-113 (2)	UGAAUUCCUCUGGCUGAAAAU GACUUGUGC	84	0.083
8-60	UGAAUUCCUUUGGCUGAAAAU GACUUGUGC	85	ND
11-221	GCAGAGCGAAAAUCGUCUUGUCCCCGACGC	86	0.062
ORPHANS			
11-123	GUGACUCAAAAUGGUGAUCCUCGUUUCGC	87	0.090
8-151	AGGACUAAUCCCUAAGGAAUAGCUUGCCCG	88	8
8-174	UCGAGCUUCUGAGUUAAA CUGGGGCCUCCU	89	0.230
8-160	GUCCCCGAAUUUAAAGUGCGUUUUCCUCCGGG	90	0.150
11-203	GGUUUUUCUUUUCUUGUUCUCUUCUUUCCCC	91	0.260
11-224	ACAGCGGCGACUAGCCUGUUCAUGCCUGCC	92	0.110
11-107	GUUCUGUGUGUCCACGUUCUUACCCCUGUG	93	0.140

[a]Clone series 8 is from HGF SELEX 1; series 11 is from HGF SELEX 3. Numbers in parentheses refer to repeat occurrences of the same exact sequence. For the series 8 clones, a second number refers to an exact match which was isolated in series 11.

[b]N7 fixed sequences are not shown. (5'-GGGAGGACGAUGCGG-N-CAGACGACUCGCCCGA-3') (SEQ. ID NO.: 2)

[c]ND, not determined.

Table 3. HGF 30N8 aptamer sequences and binding affinities.

Seq. no.[a]		30N8 random region[b]	SEQ. ID. No.	K_d (nM)
10-28		CCUGUUCUGAAC GCAAAAUGGCGUGGUGGC	94	0.860
10-40		UGUCGUUAGUUUAUUGACAAGGCCCGAAG	95	0.350
10-52		UCUUAUUGUGUCCAGCUUCUCCCUGCAGGC	96	0.160
10-72		UGUGGCAC UGUUGUCCACAAGGGCCUCA	97	0.450
10-8		UUGACAAGGUACCUGUUGCCUGGCGUUUCU	98	0.920
10-76		AGUUAGGCUUUAAAGC ACG AUAAUCAGCA	99	0.170
10-47		GUCAAGAGG AAAUGACACGG CUCCACUUUUA	100	0.390
10-2	(10)	GCCUGAGUUAAACAUGACGG UUUGUGACCC	101	0.069
10-3		GCCUGAGUUAAACAUGACGGGUUUUGUGACCCCU	102	0.072
10-23	(4)	GUCUGAGUUGGACACAACGC AUUGAGACCC	103	0.330
10-24		GUCUGAGUUgGUCACAACGC AUUGAGACCC	104	ND[c]
10-37		GUCUGAGUCCGU AGGGCGA UUUGUGUCCC	105	3.05
10-7		UGCCUUAAGAGCGGAA CUCCCUGACCCACC	106	1.45
10-13		GAUCUGUUGGCGU GU CUACCCGACCCUCCU	107	0.720
10-17		AACCCUGUUGGCGU GA CGUCCCGACCCUCC	108	0.560
10-36		CGUUAGCAUCUGAACGAUGCCCAGCCUCAA	109	1.94
10-62		GUUAGACUCAACAUGAGUCCCAGCCUCAA	110	0.440
10-29		UCUGUUGGCGUCGU UCUCCUGACCCUCCUC	111	1.75
10-48		GAGUUCCCUGUUGAC UCGC UCUCCUGACCC	112	0.310
10-16		UACAGCGUGUUGGUCCCGGACGGGGACUUAU	113	0.210
10-11		CGCCUGGACCGUUUGUUUAUCCCCGUAGUC	114	0.610
10-18		CGUGAUUCCUACCAUCA GGUACCUAUCUUG	115	0.300
10-1	(2)	AGUGAUGUGAGAG CGUGCCUCUAGUCGGUG	116	0.094
10-57		CGAGCCUCCUACCGUUU AGGUACC AUCUUG	117	0.140
10-27		UUAGCCUCCGACCG UAA GGUCCUUUUCUUG	118	0.830
10-53		GGCCUCCAACCGCUAAA GGUUCCAUUCUUG	119	0.310
10-49		CCCGACCUCCUGUAACUGGUUGA GGCACUA	120	0.240
10-31	(2)	GGGUUCCUGAUUGACCCUGUCUCUAGACCC	121	1.90
10-58		GGGGAGGCCCUUCAGCCGUCUCCUUGACCC	122	0.440
10-63		UGUGAUGUGAGGGC GUGCUUCCUAACGGUG	123	0.190
		40N8 "hitchhiker" sequences		
10-19		UUCAUUAUGCAUCGAACAGUAUACCACAGGUGUUCAUGUG	124	ND
10-35		AUCCAAAUUCUGGUCAUGAGGCGCUGCAGAUACUGCUGCG	125	2.33
10-38		UCUGCGGACGGUGAGGUUAAGUUGCAACGACUGCUUGGCG	126	7.38
10-42		CAGACCGUGCAAACCCCCCUUAGAGGGUUUUGUCAUUUAC	127	ND
10-56		CCUUAGGGCUCCCAAAAAUCGGGCC CGUCGGGCCGAUCAC	128	0.280
10-68		CGCGGGAUUCUCUGAGGACGAGGCACGUGUGGGUAAUUCG	129	1.00
10-67		UCGGGCUUGGAUGUGGACGUGUAUUUCUAGCUGUGUACGC	130	0.640
10-4		UUGGGUCGGGACUCGAAAGGAUUUGAUAGGAUACAUGAAU	131	0.610

[a]Clone series 10 is from HGF SELEX 2. Numbers in parentheses refer to repeat occurrences of the same exact sequence.

[b]N8 fixed sequences are not shown. (5'-GGGAGAUAAGAAUAAACGCUCAA-N-UUCGACAGGAGGCUCACAACAGGC-3') (SEQ ID NO.: 6)

[c]ND, not determined.

Table 4. List of HGF aptamers and their binding affinities which were tested *in vitro* for inhibition of activity.

Seq. no.	random region	K_d (nM)
"consensus"	CGUGAGCCUAUUUAUGUCAUCGU-C-UG	
8-17	GCGGCU CGAUG UCGU CUUAUCCCUUUGCCC	0.095
8-102	CGAGUGCCUGUUUAUGUCAUCGUCCGUCGU	0.060
8-104	GCGACUCAAUCUGAAUCGUCUUGUCCCGUG	0.050
8-112	UCCCGAAUUUAAGUGCGUU UCCUCCGCGUC	0.130
8-113	UGAAUUCCUCUGGCUGAAAAUGA CUUGUGC	0.083
8-122	CGGUGUGAACCUGUUUAUGUCCGCGUACCC	0.097
8-126	GUGACUCAAAAUGGUGAUCCUCG UUUCCGC	0.099
11-8	UCAGUAUGACU UUUAUAGCA CGUUCGCCC	0.150
11-76	UCAGCGGCGCGAGCCUGUUUAUGUC UGCUG	0.076
11-166	GUUUGAG UUCUGACA CGUCU UGUCCCAUGC	0.079
11-208	GUUUGAG UGACG AUCGUCU UGUCCCAUGUG	0.060
11-222	ACAUAAGUCU UCUAUAGC UCGUCCUUUGUG	0.077
10-2*	GCCUGAG UUAAACAUGACG GUUUGUGACCC	0.069
8-151	AGGACUAAUCCCUAAGGAAUAGCUUGCCCG	8

*10-2 contains N8 fixed sequences; all others are N7.

Table 5. HGF truncate SELEX 30N sequences.

Trunc Seq #[a]	# of hit	Sequence of random region (G)G-30N-CA	Identity to full-length[b]	K_d (nM	SEQ. ID. No.
		GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCC	NX22354	0.1	13
Tr7	(5)	CGGUGUGAACCUGUUUAUGUCCGCGUACCC	8-122	0.67	132
Tr45	(3)	UGGGAACCUAUUUAUGUCAUCUCCGUCCC	11-202	1.7	133
Tr70		UGGGAACCUAUUUAUGUCAUCGUCUGUGCC	New	2.4	134
Tr6		CGUGAGCCUAUUUAUGUCAUCAUGUCUGUG	8-114	9.0	135
Tr20		UGUGAACCUGUUUAUGCCAUCUCGAGUCCC	New	3.4	136
Tr23		UGUGAACCUAUUUAUGCCAUCUCGAGUGCC	8-155	ND[c]	137
Tr42		UGAUAACCUAUUUAUGACGUCGUGGCUCCC	11-162	6.1	138
Tr44		AGUGAUCCUAUUUAUGCCGUCGCUUCUCGC	New	6.5	139
Tr65		AGAGNUCCUAUUUAUGACAUCCCAUGCCCC	New	1.4	140
Tr48		UGAUCACCUGUUUAUGCCAUCGUUCUGGGC	11-65	1.8	141
Tr28		GGUGACCCUUUUUAUGACAUCGCGUCUGGC	New	4.0	142
Tr51	(6)	AAUCACAGGAAUCAACUUCUAUUCCCGCCC	New	0.06	143
Tr67		AAUCACAGGAAUCGACUUUUAUUCCUGCCC	New	ND	144
Tr17		GC GGCUCGAUGUCGUCUUAUCCCUUUGCCC	8-17	3.0	145
Tr27		UC GGCUCGUUGUCGUCUUAUCCCUUUGCCC	New	ND	146
Tr18		GCUGGCUCGAUGUCAGGUUAUCCCUUUGCCC	New	ND	147
Tr4	(4,2)[d]	GUGACUCAAAAUGGUGAUCCUCGUUUCCGC	8-126	1.4	148
Tr31	(2)	UGAAUUCCUCUGGCUGAAAAUGACUUGUGC	8-113	9.2	149
Tr15		GUUUGAGUGACGAUCGUCUUGUCCCAUGUG	11-208	8.8	150
Tr1		AUUGAUUCACUGCAUCCUUGACUCUUCCCC	New	7.3	151
Tr5		CAGACGACUCGCCCGAAGGACGAUGCGG	New	28	152
Tr14		GAGUUAUAUUUCGUCACCCGUUCCUUUGCCC	New	2.2	153
Tr59		ACAGUUUGUCUUCUAUAGCUCGUCGCCCC	New	7.2	154
Tr71		UCAGAAUGACUUUCAUAGCUCGCUUUCCCC	New	7.7	155

[a]Tr1-36 and Tr37-72 clones are from series which were carried through 8 and 11 conventional rounds, respectively.

[b]Sequences indicated are identical to full length aptamers derived from series 8 or 11; NX22354 is a synthetic truncate based on boundary experiments, derived from sequence 8-102, shown here for comparative purposes.

[c]ND, not determined.

[d](4,2) refers to 4 occurrences in the first series and two in the second series.

Table 6. Invasion of A549 cells through Matrigel is inhibited by HGF aptamer NX22354.

Sample	HGF 10 ng/ml	Inhibitor	Cells migrated
1	-	-	40
2	+	-	240
3	+	mAb[a], 1 μg/ml	120
4	+	NX22354, 1 uM	40
5	+	NX22354, 0.2 uM	25
6	+	NX22354, 0.04 uM	200

[a]Anti-HGF antibody was MAB294 from R&D Systems, Inc.

Table 7. Partially 2'-O-methyl substituted variants of NX22354.

	SEQUENCE	SEQ. ID. No.
NX22354 (parent)	GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCC *** ** * ** *** * * * * * * *	13
HGFOMe1	GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCCg	156
HGFOMe2	GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCCg	157
HGFOMe3	GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCCg	158
HGFOMe4	GGACGAUGCGGCGAGUGCCUGUUUAUGUCAUCGUCCg	159

Parent 36mer sequence of NX22354 (purines marked with asterisks).
The substituted positions are indicated by underlines. The OMe1 sequence has five substitutions while the others have four. For technical reasons, a G residue was added at the 3'-end of each aptamer.

Table 9. 40N7 sequences isolated from a plate SELEX on human c-met.

Clone name:	(number of isolates).	Sequence[a]	SEQ ID NO:
FAMILY 1:			
7C -1:	(2)	UUUGACUAUGUCUGACGGGUCUGUGGUCAAUUCCGCCCC	160
FAMILY 2			
7C -4:	(1)	AUCCGUGUUGAUGUCCAUAUAACCUUAUCCCGUCGCUCCC	161
7C -5:	(1)	GUGUUGACUUCUAGCCAGAAUAACAUUUUGUACCCCUCCC	162
FAMILY 3			
7C -2:	(1)	UCGUUGAGCUUUUGAUAGGGCUUGUUCUUCGAGCGUCCC	163
7C-23:	(1)	UGAUCUUGGGUUUGAUCGUAAUUACUUCACCCUCCGUCCC	164
FAMILY 4			
7C-26:	(2)	CUCCUUUUCCGCUAAACAAGACCACUUUGAGCCCUGCCCC	165
FAMILY 5			
7C-25:	(1)	CCACCUCGUUACGUACUGAUUUUGGCAUCGCAGUUUGCCC	166
7C-27:	(1)	GGGCACCUCGAUACGUACUGAUUUUGAAUAUCAGUUAGCCCC	167
OTHERS			
7C-21:	(1)	CGAUUCGUCGUAUAGAAAUGAUUUGAAUGCACCUCCUCCC	168
7C-24:	(1)	UGUGUUUGUGUGUUGUGUUUGUUAUUCCUGUUUGUGUCCU	169
7C-32:	(1)	UCGGUCGUAAAAAAUCGUUGGUGUCUAUCUAUUGUUCUCCC	170
Presumed IgG_1 aptamers			
7C -3:	(1)	UGCUCCAGAGGAACCAUCGUUUACUUCAUUUAUUCGCCC	171
7C-22:	(1)	UGCUCCUUAGGAACCAUCGUCUAUAUCCCAUUCUGACUGCC	172
7C-30:	(1)	UGCUCCUCAGGAACCAUCGUUUUUCCCAUGUCCUUCUGCC	173
7C-29:	(3)	UGCUCCUUGGAUUACCAAGGAACCAUUUUCCUCUACCCCC	174

[a]N7 fixed sequences are not shown. (5'-GGGAGGACGAUGCGG-N-CAGACGACUCGCCCGA-3') (SEQ ID. NO: 2)

Table 10. 30N8 sequences isolated from a plate SELEX on human c-met.

Clone name: (number of isolates).	Sequence[a]	SEQ. ID. NO:
FAMILY 1:		
7b-1: (4)	GUGCUCAUUACGAACUUGACCGAUGCCUA	175
7b-9: (1)	GGUGCUCAUUACGAACUUGACCGAAGCCUA	176
7b-18: (1)	GGUGCUCAUUACGAACUUGACCGAUGCCUA	177
7b-3: (1)	AGUGCUCCAAUGAACUUUGCUCGCUGA	178
7b-8: (1)	GGUGCUCCGUUUGGAACUUGAUCGGUAGGA	179
7b-7: (1)	GUGCUCAUUCAGAACUUGACGUAUAACCA	180
7b-14 (1)	GGUGCUCCUUAGGAACUUGACCGUCCGCCA	181
7b-16: (1)	GUGGUGCUCCACUAACCAAGUGGAACCUUG	182
consensus:	GUGCUC-UU--GAACUUGACCG	183
OTHERS:		
7b-10: (1)	ACGAUAAGUGGGAGUGAGUAAGUUUGAGUA	184
7b-12: (1)	CCUAGACCCCCAGGUUCCUCCCCACUAGUC	185

[a]N8 fixed sequences are not shown. (5'-GGGAGAUAAGAAUAAACGCUCAA-N-UUCGACAGGAGGCUCACAACAGGC-3') (SEQ. ID NO.: 6)